LncRNA TTN-AS1 intensifies sorafenib resistance in hepatocellular carcinoma by sponging miR-16-5p and upregulation of cyclin E1

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ABSTRACT

Drug resistance has always been an important problem affecting the therapeutic effect of hepatocellular carcinoma (HCC). To investigate the potential role of lncRNA TTN-AS1 in HCC cells with sorafenib (SOR) resistance, and explore the underlying pathways, quantitative real time polymerase chain reaction (qRT-PCR) was used to test the expression of TTN-AS1 in HCC tissues and cells. Then, the expression of TTN-AS1 was down-regulated by shRNA, the activity changes, apoptosis and related protein expression in HCC cells with/without SOR treatment were observed in succession. Expression levels of the downstream target of TTN-AS1, miR-16-5p were studied by dual-luciferase binding assay, cell proliferation, and western blotting analysis. Nude mice models of human HCC with TTN-AS1 gene knockdown were established to observe the tumor growth. As the results revealed, TTN-AS1 silencing in HCC cells induced apoptosis by enhancing the sensitivity of cells to SOR, and the tumor in nude mice became smaller. The mechanism study showed that miR-16-5p was affected by TTN-AS1 sponge, up-regulated cyclin E1 expression, and regulated PTEN/Akt signaling pathway, thereby significantly alleviating the inhibition of apoptosis of HCC cells induced by TTN-AS1 gene. Collectively, our results provided TTN-AS1 as a potential therapeutic target for sorafenib resistance in HCC.

1. Introduction

Hepatocellular carcinoma (HCC) is a kind of aggressive cancer with high incidence and mortality [1,2]. It is also one of the most common fatal digestive tract tumors [3], and the third most fatal type of cancer globally after gastric cancer and lung cancer [4]. Reducing the mortality of HCC and preventing its malignant prognosis is still urgently needed. However, the prognosis of chemotherapy is poor and there is no effective follow-up therapy [5]. In clinical practice, sorafenib (SOR) is currently the most commonly used first-line targeted multi kinase inhibitors approved for the treatment of advanced diseases, while the average survival time of the treated patients increased from 8 months to 12 months [6–9]. More and more clinical trials have found that the drug resistance of HCC cells under the treatment of SOR has been gradually produced, thus reducing the quality of life of patients [10,11]. Some studies have shown that HCC patients treated with SOR are often accompanied by the chemical resistance effect and poor prognosis [12].
Therefore, exploring the mechanism of SOR and developing alternative therapies for HCC is extremely urgent.

Long noncoding RNAs (LncRNAs) are a newly discovered regulatory class of ncRNAs in recent years [13]. They are transcripts with more than 200 nucleotides in length, few introns as well as open reading frames, and have no protein coding ability [14]. According to the genome location, lncRNAs are generally divided into five types: justice type, intron type, antisense type, intergenic type and bidirectional type [15]. Recent studies have shown that lncRNAs are involved in many biological processes, including cell differentiation, proliferation, migration, infiltration, apoptosis and autophagy [16]. The expression of lncRNAs is tissue-specific and dysregulated in many types of tumors, which is related to tumor recurrence and poor prognosis [17]. These lncRNAs work by recruiting specific proteins or participating in microRNA (miRNA) regulatory networks. Studies revealed that lncRNAs are of vital importance in regulating the proliferation, apoptosis, migration, invasion and drug resistance of tumor cells [18–21]. For example, Cheng et al. found that lncRNA NCK1-AS1 acted as a NCK1-AS1 silence oncogene and reduced the DDP resistant of osteosarcoma cells [22]. Jiang et al. found that inhibition of lncRNA MALAT1 can induce apoptosis and metastasis of laryngeal squamous cell carcinoma (LSCC) cells, and enhance the sensitivity to chemotherapy drugs [23]. Xue et al. found that lncRNA SSTR5-AS1 facilitated gemcitabine resistance *via* stabilizing NONO in gallbladder carcinoma [24]. Therefore, developing the key lncRNA to regulate the drug resistance of tumor cells is the driving force to promote the progress of tumor treatment.

TTN antisense RNA 1 (TTN-AS1) is a kind of lncRNAs which is transcribed from the relative chain of titin gene. Its tumor promoting effect has been found in many kinds of cancers [25]. For example, TTN-AS1 promotes cell proliferation and inhibits cell apoptosis in prostatic cancer by sponging miR-193a-5p [26]. TTN-AS1 regulates osteosarcoma cell apoptosis and drug resistance *via* the miR-134-5p/MBTD1 axis [27]. In addition, TTN-AS1 is also involved in the progression and metastasis of ESCC. And the potential function of TTN-AS1 in HCC has been confirmed in the past [28]. Yet, the role of TTN-AS1 in drug resistance of HCC cells remains largely unknown.

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Previous studies confirmed that miRNAs are involved in various biological processed in HCC [29]. LncRNAs, mRNA transcripts can affect each other by competitively combining with miRNA response sequence to influence post-transcriptional regulation. In present study, we first identified the promoting-effect of lncRNA TTN-AS1 on sorafenib-resistance in HCC both *in vitro* and in *vivo*. And we also confirmed miR-16-5p was a direct target of TTN-AS1, Cyclin E1 as the target of miR-16-5p. The effects of miR-16-5p and Cyclin E1 on the biological behavior of sora-resistance in HCC also explored *in vitro*. Knockdown of TTN-AS1 could enhance the sensitivity of HCC cells to SOR, while inhibition of miR-16-5p and overexpression of Cyclin E1 could reverse this sensitivity caused by knockdown of TTN-AS1. Therefore, this study was to investigate the role, function and mechanism of TTN-AS1 in the SOR-resistance of HCC cells and explore whether the downstream signaling pathway was *via* sponging miR-16-5p, up-regulating cyclin E1, and mediating PTEN/ Akt signaling pathway.

2. Materials and methods

The HCC cell lines Bel7404 and HepG2 were purchased from the cell bank of Chinese Academy of Sciences (Shanghai, China). Cells were grown in Dulbecco's Modified Eagle's medium (DMEM, hyclone) with 10 % FBS (GIBCO), 100 U/mL penicillin and 100 mg/mL streptomycin (GIBCO). The cells were cultured with 5% CO_2 at 37 ℃. All animal experiments in this study were preapproved by the ethics committees of the affiliated Wuxi Second Hospital of Nanjing Medical University and The Affiliated BenQ Hospital of Nanjing Medical University.

2.1. HCC cells transfection

The plasmids of TTN-AS1 complementary DNA (cDNA), pcDNA-3.1, miR-16-5p mimics, and miR-NC were obtained from Genechem (Shanghai, China). The oligonucleotide sequences were: sh-TTN-AS1, sense: 5′-CCAGACACCTAACCAACTTCC-3′; NC, sense: 5′-CGATCGCATCAGCATCGATTGC-3′. miRNA mimics negative control (miR-NC), miR- 16-5p inhibitor and miRNA inhibitor negative control (anti-NC) were synthesized by GenePharma Co. Ltd (China, Shanghai) for constructing overexpression or inhibition of miR-16-5p, miR-16-5p mimics. The sequences were: miR-16-5p mimics, sense: 5′-UAGCAGTTGGUAAAUAUUGGCG-3′; miR-NC, sense: 5′-UUCUCCGAACGUGUCACG UTT-3′; miR-16-5p inhibitor, 5′-CGCCAAUAUUUACGUGCUGCUA-3′; anti-NC, 5′-CAGUACUUUUGUGUAGUACAA-3′. The small interfering RNA (siRNA) and the negative control (NC) oligonucleotides were purchased from Sigma-Aldrich (Genechem, Shanghai, China). The plasmids and siRNA were transfected into cells with Lipofectamine 3000 (Invitrogen) according to the manufacturer's instructions.

2.2. Cell counting kit-8 (CCK-8) assay

Relative cell viability was assessed using CCK-8 assay according to the procedure previously described with minor modification [30]. After transfection of shRNA and miR-16-5p inhibitor with or without the action of 10 μM SOR, HCC cells Bel7404 and HepG2 (5×10^3 cells/well) were cultured in complete DMEM medium, and then harvested and washed twice using phosphate-buffered saline (PBS) for another 48h-culture. After giving a total of 10 μL CCK-8 to each well, the microplate was incubated at 37℃ for 1 h. The optical density (OD) at 450 nm was read with a 96-well multi-scanner plate reader (Biotech Instruments). Experiments were conducted with 3 replicates.

2.3. Flow cytometry

To evaluate the effects of LncRNA TTN-AS1 on apoptosis and cell cycle changes in HCC cells, the flow cytometry was applied. Detailedly, Bel7404 and HepG2 cells were cultured at 37 °C in the dark condition for 10 min and then were collected. The survival status of Bel7404 and HepG2 cells was detected by annexin V-FITC (AV) and PI apoptosis detection kit (Solarbio, USA) or cell cycle detection kit (KeyGEN BioTECH, China). Specifically, the cell suspension (5×10^5/mL, 100 μl) was incubated with 5 μl AV and 5 μl PI (apoptosis) or PI/RNase A (cycle) respectively at room temperature in the dark for 15 min. After adding binding buffer 400 μl/tube, samples were evaluated with flow cytometry (BD Accuri™ C6, USA).

2.4. RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

As mentioned in the literature, the gene expression levels of Bel7404 and HepG2 cells were detected by quantitative real-time PCR (qRT-PCR) [31]. Specifically, after centrifugation, cells are collected, total RNA was extracted under the action of TaqMan gene expression Kit (Applied Biosystems, Carlsbad, CA), and cDNA was prepared according to the manual. The heat start enzyme was activated at 95 ℃ for 5 min and amplified 40 cycles of cDNA, including 15 s denaturation at 95 ℃ and 30 s annealing/elongation at 58 ℃. Then, melt curve analysis (55 °C for 1 min, then 0.5 °C for every 10 s) was carried out to detect the formation of primer derived modifiers and dimers. GAPDH was used as the internal control for TTN-AS1 and cyclinE1. The expression of miR-16-5p was measured using TaqMan MicroRNA Assays and U6 as its internal control. Each sample was tested three times for statistical analysis. The relative expression was calculated by $2^{-\Delta\Delta Ct}$ method.

2.5. Dual-Luciferase reporter assay

The dual-luciferase activity assay was performed as described [32]. The wild-type binding sites of TTN-AS1 with miR-16-5p binding site (WT TTN-AS1), the mut-type binding sites of TTN-AS1 with miR-16-5p (MUT TTN-AS1), the wild-type binding sites of cyclin E1 with miR-16-5p (WT cyclin E1) and the mut-type binding sites of cyclin E1 with miR-16-5p (MUT cyclin E1) were cloned downstream of the luciferase gene coding sequence. The recombinant plasmids were co-transfected with miR-16-5p mimic and miR-NC into HepG2 cells. The dual luciferase

assay was done by using the Dual-Glo Luciferase Assay System Kit (E2920; Promega) according to the instructions. The luciferase activity of firefly was normalized to that of Renilla.

2.6. Western blotting analysis

Before protein extraction, the homogenization of the collected cells was first done and the total protein lysates were prepared. Subsequently, we resolved the lysates in lysis buffer (10 % SDS-PAGE). When the lysates were completely resolved, the solution was loaded to polyvinylidene fluoride membranes (MilliporeSigma, Burlington, MA, USA). For the blocking step, the buffer we used was 5% skim milk powder in Tris-buffered saline containing 0.1 % Tween-20 (TBS-T) and the blocking time was 30 min. After washing, the membranes were washed four times with TBS-T and then incubated with primary antibody against cleaved caspase 9, Bax, Bcl-2, PTEN, p-Akt, Akt, and GAPDH that with the dilution times of 1:1500 (Cell Signaling Technology, Danvers, MA) at 4 °C overnight. Immunoreactivity measurement was done by using an Enhanced Chemiluminescence (ECL) Kit (Pierce Biotechnology, Waltham, MA, USA). The relative expression level of target protein was quantitatively analyzed by gray value integration.

2.7. In vivo tumorigenesis

Adult male Balb/c nude mice (4–6 weeks old) were purchased from Cavens Laboratory Animal Company (Changzhou, China). All mice were randomly divided into three groups: control, SOR, and SOR + sh-TTN- AS1 (N = 3 each). The control group received subcutaneous injection of HepG2 cells (10^7, 100 μ L) and SOR + NC group. SOR + sh-TTN-AS1 group received HepG2 cells transfected with sh-TTN-AS1. When the tumor in the blank control group grew to approximately 0.6 cm in diameter, a total of 30 mg/kg SOR was injected into the abdominal cavity of mice every other day. After 24 days, the mice were euthanized, the tumor was dissected, measured, weighed, and analyzed by qRT-PCR and Western blotting with careful records. The experiments were repeated three times independently. The following formula: $V = a \times b^2/2$ (mm^3) was used for tumor growth monitoring, in which “a” was the maximum diameter and “b” was the vertical diameter.

2.8. Statistical analysis

All data were performed as mean ± SD. The student *t*-test was used for the analysis of two independent groups. The one-way ANOVA and Tukey test were applied to analyze the differences among three groups or more. $P < 0.05$ was statistically significant.

3. Results

3.1. Knockdown of lncRNA TTN-AS1 suppresses SOR resistance of HCC cells by inducing cell apoptosis

In order to explore the effect of TTN-AS1 on the survival of HCC cells, shRNA was used to inhibit the expression of TTN-AS1 in HepG2 and Bel7404 cells. The results of qRT-PCR showed that the expression of TTN-AS1 in HepG2 and Bel7404 cells was significantly down regulated after shRNA transfection, $P < 0.001$ compared with NC group (Fig. 1a). The two hepatoma cell lines were co-incubated with 10 μM SOR, and the changes of cell viability were detected by CCK-8 assay. The results showed that inhibition of TTN-AS1 expression significantly enhanced the sensitivity of hepatoma cells to SOR and induced apoptosis (Fig. 1b). Flow cytometry also showed that the apoptotic level of these two cells increased with the down-regulation of TTN-AS1 expression (Fig. 1c, d). We further studied the expression of apoptosis related proteins, and found that the expression level of caspase-9 and Bax in apoptotic cells increased significantly, while the expression level of Bcl-2 in apoptotic cells decreased significantly. At the same time, this phenomenon is further strengthened after sh-TTN-AS1 operation (Fig. 1e). The above results indicate that TTN-AS1 is an important target to regulate the resistance of HepG2 and Bel7404 cells to SOR.

3.2. LncRNA TTN-AS1 sponges miR-16-5p expression

More and more studies have shown that lncRNA can play a role by regulating miRNA. In this study, we used TTN-AS1 as the target to study the miRNA molecules targeted and regulated, and elucidated the mechanism of its mediating the resistance of HCC cells to SOR. The intersection of prediction targets using miRanda, LncBase and Starbase databases showed that there were continuous TTN-AS1 wild type binding sites on the miR-16-5p sequence (Fig. 2a). Some reports have proved the proliferation- and metastasis-promoting effect of miR-16-5p in HCC [33,34]. First, online prediction showed that there was a binding site between TTN-AS1 and miR-16-5p. Therefore, we mutated the corresponding binding sequence in TTN-AS1 to construct a mutated plasmid mut TTN-AS1 for subsequent cell transfection. The results of double Luciferase Report showed that compared with the control group, the fluorescence intensity of wild-type TTN-AS1 changed significantly, while that of mutant plasmid transfection did not change significantly (Fig. 2b). Further study on the relationship between TTN-AS1 and miR-16-5p expression levels in HepG2 and Bel7404 cells showed that the expression of miR-16-5p was down regulated after overexpression of TTN-AS1 (Fig. 2c), and the expression of miR-16-5p was up regulated after downregulation of TTN-AS1 (Fig. 2d). That is to say, there is a negative correlation between TTN-AS1 and miR-16-5p.

3.3. Knockout of miR-16-5p enhanced the SOR resistance of sh-TTN-AS1-HCC cells

Next, we investigated whether miR-16-5p mediates the resistance of TTN-AS1-induced hepatoma cells to SOR. MiR-16-5p expression in both HepG2 and Bel7404 cells of sh-TTN-AS1 was inhibited (Fig. 3a), and then the effect on SOR induced HCC cell viability was detected. The results showed that inhibition of miR-16-5p significantly increased the resistance of HepG2 and Bel7404 cells to SOR, and the apoptosis rate decreased from 70% to 30% (Fig. 3b–d). Moreover, after knockdown of miR-16-5p, the cleaved caspase-9 and Bax showed a low expression, and the anti-apoptotic factor Bcl-2 demonstrated a high expression (Fig. 3e).

3.4. miR-16-5p modulates cyclin E1 expression

In order to clarify the specific relationship between miR-16-5p and the sensitivity of HCC cells to SOR, we further studied the downstream target gene. Based on the intersection of targets predicted by miRmap, miRanda and TargetScan, and it was found that there was a binding site between miR-16-5p and cyclin E1. There also were studies demonstrated that cyclin E1 was closely related to drug-resistance in HCC [35,36]. Then we mutated the corresponding binding sequence in cyclin E1 to construct a mutated plasmid mut cyclin E1 for subsequent cell transfection (Fig. 4a). The results of double Luciferase Report showed that there was a binding between miR-16-5p and 3′-UTR of cyclin E1 (Fig. 4b). The results of qRT-PCR and Western blotting suggested that inhibition of miR-16-5p

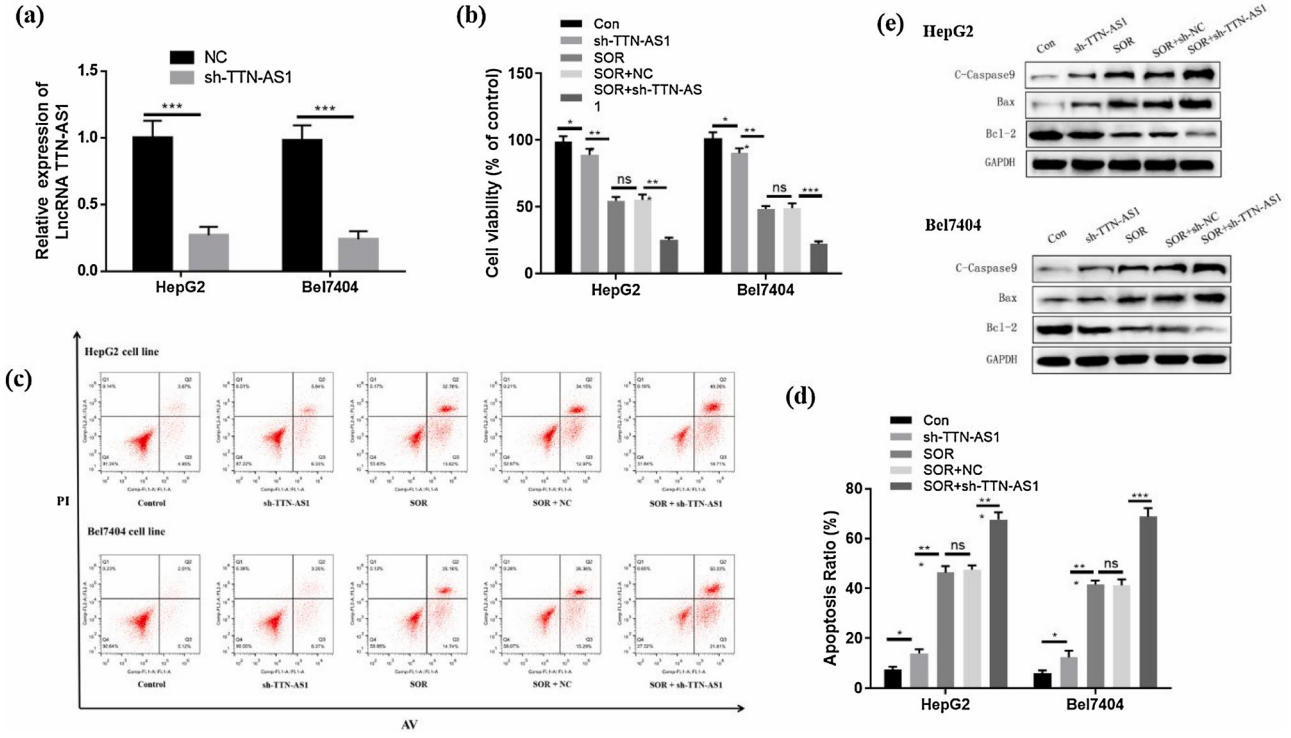

Fig. 1. LncRNA TTN-AS1 knockdown facilitated SOR sensitivity of HCC cells. (a) qRT-PCR for TTN-AS1 expression across NC and sh-TTN-AS1 groups, NC means negative control of shRNA. (b) Relative cell viability of HepG2 and Bel7404 HCC cells with TTN-AS1 knockdown after SOR treatment. (c, d) Flow cytometry assay for apoptotic levels of cancer cell lines. (e) Western blot assay for quantification of cleaved caspase 9, Bax, and Bcl-2 levels. ns. no significant difference, *$P < 0.1$, ** $P < 0.01$, *** $P < 0.001$.

significantly increased the expression level of cyclin E1 in HepG2 and Bel7404 cells (Fig. 4c, d).

3.5. LncRNA TTN-AS1 intensifies sorafenib resistance in HCC by sponging miR-16-5p and upregulations of cyclin E1 via regulating PTEN/ Akt signaling pathway

Combined with the above results, we knocked out TTN-AS1 as well as miR-16-5p, and overexpressed cyclin E1 in both HepG2 and Bel7404 cells. The results of cell viability showed that inhibition of miR-16-5p or overexpression of cyclin E1 could eliminate the enhanced SOR sensitivity after TTN-AS1 knockdown (Fig. 5a). Flow cytometry results demonstrated that inhibition of miR-16-5p or overexpression of cyclin E1 could alleviate the apoptosis of HepG2 and Bel7404 cells with sh- TTN-AS1 (Fig. 5b, c). Due to cyclin E1 is one protein related to cell cycle, the cell cycle affected in different groups was also determined by flow cytometry. Results as shown in Fig. 5d, inhibition of TTN-AS1 could arrest cells in G0/G1 phase, while inhibition miR-16-5p or overexpression of cyclin E1 could alleviate the arrest caused by TTN-AS1 and promote cells to S phase. Western blotting analysis further showed that repressed expression of miR-16-5p or cyclin E1 overexpression might be responsible for the inhibition of lysed caspase-9 and Burlington levels, while geographic Bcl-2 protein expression increased, and could also partially rescueTTN-AS1 to knock down the induced upregulation-9 or Burlington of HCSE, reducing Bcl-2, HepG2 or Bel7404 cells (Fig. 5e).

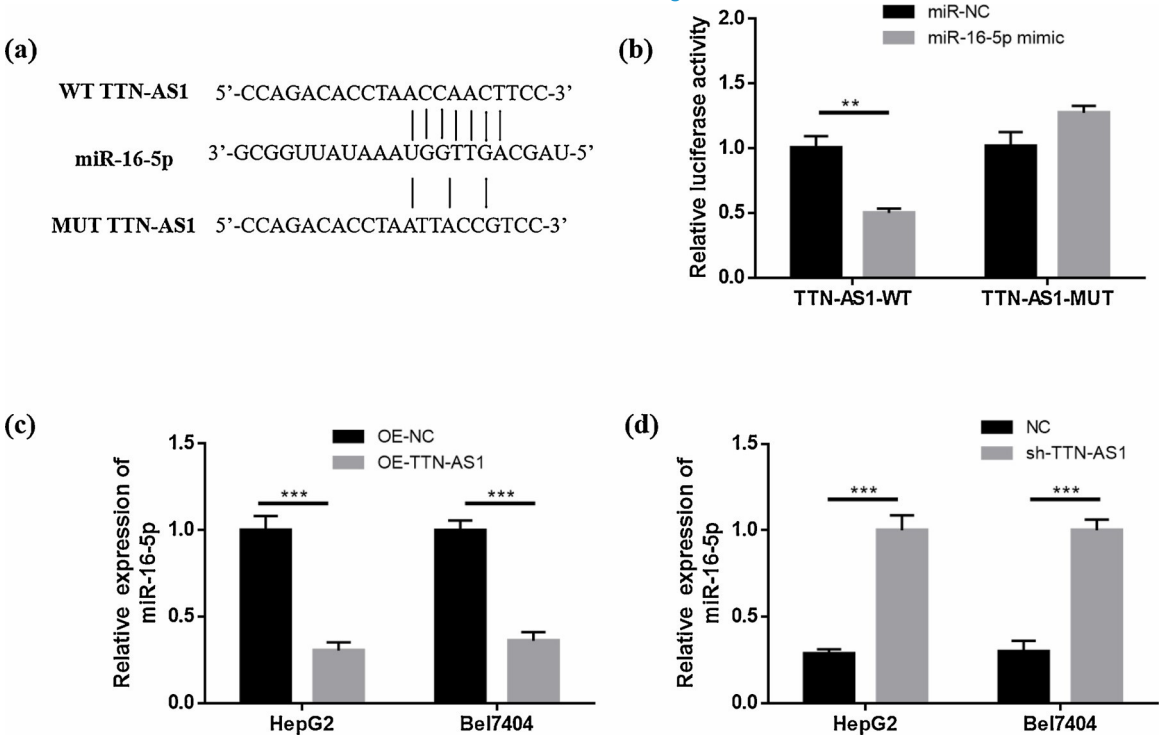

Fig. 2. LncRNA TTN-AS1 sponged miR-16-5p expression. (a) Online prediction of miR-16-5p binding sites with TTN-AS1. (b) Dual-luciferase reporter assay. (c)
Relative expression level of miR-16-5p in HepG2 and Bel7404 cells after TTN-AS1 overexpression. (d) miR-16-5p expression level after TTN-AS1 knockdown. ** $P < 0.01$, *** $P < 0.001$.

Fig. 3. Knockout of miR-16-5p enhanced the SOR resistance of sh-TTN-AS1- HCC cells. (a) Relative expression of miR-16-5p in HepG2 and Bel7404 cells. (b) Relative cell viability of HepG2 and Bel7404 cells with miR-16-5p inhibitor transfection after the action of SOR. (c, d) Flow cytometry assay for HepG2 and Bel7404 cells apoptosis. (e) Western blotting analysis for expression of cleaved caspase 9, Bax, and Bcl-2 proteins. ns. no significant difference, *$P < 0.1$, ** $P < 0.01$, *** $P < 0.001$.

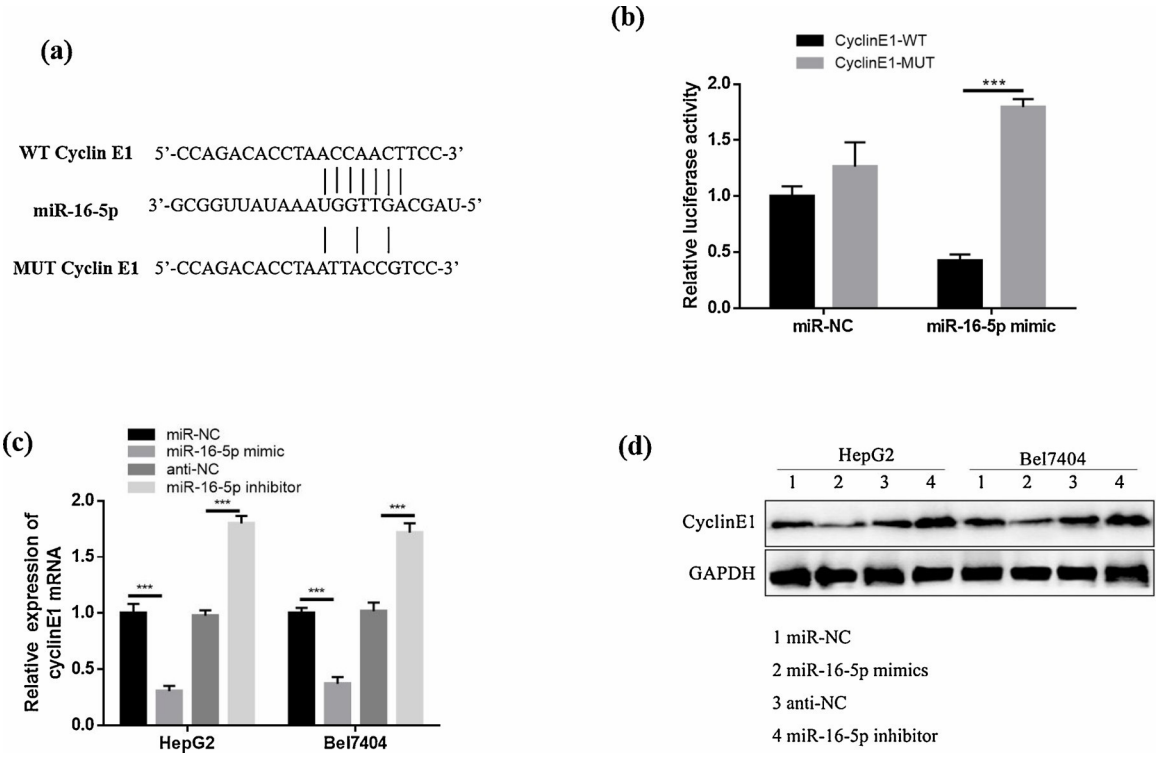

Fig. 4. miR-16-5p modulates cyclin E1 expression. (a) Online prediction of binding sites between miR-16-5p and 3′-UTR of cyclin E1 sequence. (b) Dual-luciferase reporter assay for binding affinity between miR-16-5p and cyclin E1 sequence. (c) Relative expression of cyclin E1 transcript in HepG2 and Bel7404 cells after the action of miR-16-5p inhibitor. (d) Relative expression level of cyclin E1 protein by western blotting. ** $P < 0.01$, *** $P < 0.001$.

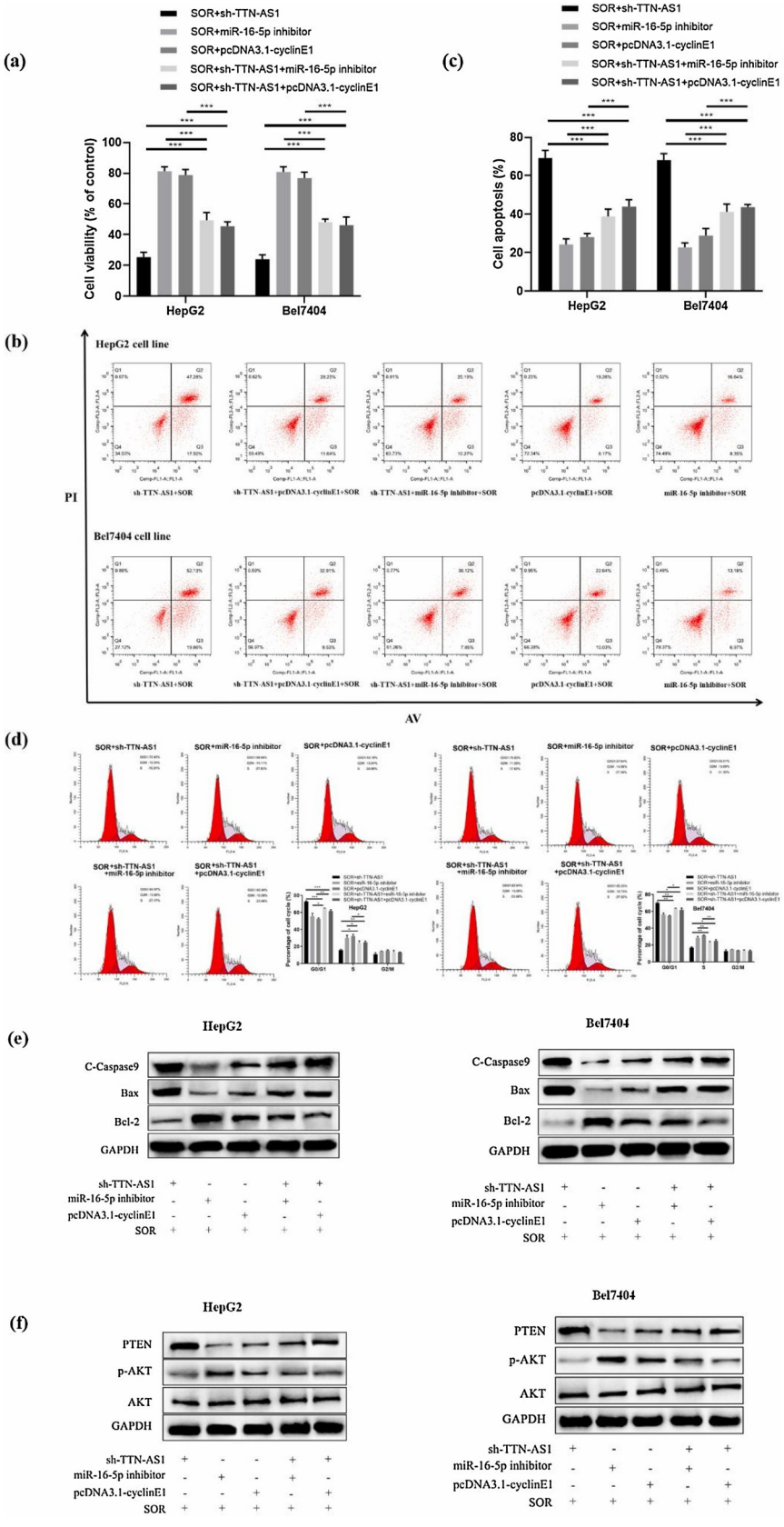

Fig. 5. LncRNA TTN-AS1 intensifies sorafenib resistance in HCC by sponging miR-16-5p and upregulations of cyclin E1 *via* regulating PTEN/Akt signaling pathway. (a) Relative cell viability of HepG2 and Bel7404 cells with sh-TTN-AS1, miR-16-5p inhibitor, and cyclin E1 overexpression. (b, c) Flow cytometry assay for HepG2 and Bel7404 cells apoptosis. (d) LncRNA TTN-AS1 knockdown facilitated SOR sensitivity of HCC cells by arresting cell cycle at G0/G1 phase in both HepG2 and Bel7404 cells. (e) Western blotting analysis for expression of cleaved caspase 9, Bax, and Bcl-2 proteins. (f) Deactivation of cyclin E1/Akt signaling pathway after the action of TTN-AS1. $*P < 0.1$, $** P < 0.01$, $*** P < 0.001$.

TTN-AS1 was reported that involved in PTEN/Akt signaling pathway [37]. And PTEN/Akt signaling pathway has been proved associated with SOR resistance in HCC [38]. Thus, the effect of TTN-AS1 on PTEN/Akt signaling pathway was explored. In-depth study of the mechanism found that TTN-AS1 effectively inhibited the activation of PTEN/Akt signaling pathway, and miR-16-5p inhibitor or overexpression of cyclin E1 reversed the inactivation (Fig. 5f). Thus, we proposed that LncRNA TTN-AS1 intensifies SOR resistance in HCC by sponging miR-16-5p and upregulations of cyclin E1 and regulating PTEN/Akt signaling pathway.

3.6. Knockdown of TTN-AS1 alleviated SOR resistance in vivo

HepG2 cells transplanted with sh-TTN-AS1 were injected subcutaneously into nude mice to form a tumor model *in vivo*, and the tumor inhibition *in vivo* was observed under different treatments. Western blotting analysis showed that compared with control group and SOR group, the expression levels of cleared caspase 9, Bax and PTEN in SOR + sh-TTN-AS1 group were significantly higher, and the expression levels of Bcl-2 and p-Akt were significantly lower (Fig. 6a), thereby indicating that knowledge of TTN-AS1 sponged SOR resistance *in vivo* by regulating PTEN/Akt signaling pathways.

The results showed that when compared with the blank control group, the tumor size and weight of the SOR group decreased, and the tumor inhibition of the SOR + sh-TTN-AS1 group was the most obvious (Fig. 6b–d), suggesting that the tumor bearing mice after TTN-AS1 gene knockout were more sensitive to SOR. We further detected the expression of TTN-AS1 and miR-16-5p in tumor tissue, and the results revealed that SOR and sh-TTN-AS1 could effectively inhibit the expression of TTN-AS1, and improve the expression of miR-16-5p compared with control group (Fig. 6e, f).

4. Discussion

HCC is one of the common clinical malignant tumors, which seriously threatens the health of our people [39]. Although new methods of clinical treatment have been developed and applied, the benefits of HCC patients are still limited. The death of primary HCC each year in China about 810000 cases, accounting for 45 %–50 % of all deaths caused by diseases [40]. Therefore, in-depth study of the pathogenesis and development of new strategies for the treatment of HCC has become a hot topic in the field of liver cancer treatment.

In recent years, lncRNAs have attracted much attention in the field of HCC treatment. More and more evidences have shown that lncRNAs and miRNAs can be used as markers of diagnosis and prognosis of HCC. As a newly discovered lncRNA, the carcinogenic effect of TTN-AS1 has been confirmed in cervical cancer [41], thyroid papillary cancer [42], lung adenocarcinoma [25] and many other human tumors. Additionally, previous studies have also highlighted the potential significance of TTN-AS1 in HCC [28].

Previous reports found that the expression of TTN-AS1 in HCC tissues and cells increased significantly [28]. TTN-AS1 is located on chromosome 2, which was first found to be related to esophageal squamous cell carcinoma. Patients with high expression of TTN-AS1 have poor prognosis and high risk of metastasis. Luo's results also showed that TTN-AS1 was highly expressed in lung adenocarcinoma and promoted the development of tumor by regulating PTEN/PI3K/Akt pathway. Recent studies have shown that TTN-AS1 can be used as Cerna targeting miRNA to promote tumor development in cervical, gastric and thyroid cancer. However, the mechanism of TTN-AS1 resistance to HCC has not been systematically studied. In order to find out, we conducted this study and found that TTN-AS1 could promote cell viability, inhibit cell apoptosis, and down-regulating TTN-AS1 could reduce the resistance of SOR. These results suggested that TTN-AS1 could promote the carcinogenesis of HCC and participate in the drug resistance of HCC.

Then, we further analyzed how TTN-AS1 achieved the function of promoting SOR resistance. Because lncRNAs often regulates the transcriptional activity of miRNA indirectly targeted genes, we screened the potential target miRNA molecules of TTN-AS1. The results of dual-luciferase report analysis indicated that TTN-AS1 targeted miR-16-5p for further treatment resistance of tumor drugs. The results showed that miR-16-5p was sponge coated in sh-TTN-AS1 HCC cells, which inhibited the sensitivity of HCC cells to SOR and increased the cell survival rate.

PTEN gene is a new tumor suppressor gene discovered in recent years, and its product PTEN protein has the activity of lipid phosphatase and protein phosphatase. PI3K/Akt signaling pathway is an important survival signaling pathway *in vivo*. It is confirmed that PTEN can inhibit the cancer by blocking the PI3K/Akt signal pathway through its lipid phosphatase activity acting on the downstream target molecule PIP3 of PI3K. In addition, a recent study found that cyclin E1 suppression by SOR in the regional carcinoma cells correlated with cellular efficacy of SOR. Furthermore, we elucidated that TTN-AS1 blocked PTEN-MAGI2 expression in HCC by promoting the interaction of PTEN phosphorylation, and TTN-AS1 can damage the stability of PTEN by activating PI3K/ Akt pathway, which may be the treatment of liver cancer patients, but more evidence should be provided in the future.

In conclusion, our results showed that inhibition of TTN-AS1 significantly increased sensitivity of SOR to HCC cells, and promoted the apoptosis as well as the arrest in G0/G1 phase of HCC cells *in vitro* and significantly enhanced the tumor-suppression effect of SOR *in vivo*. It provides a possible strategy to overcome sorafenib resistance using TTN-AS1/miR-16-5p/Cyclin E1 axis by inhibiting PTEN/ Akt pathway. It is worth to further study the efficiency of TTN-AS1 and the gene knockout therapy of drug-resistant HCC.

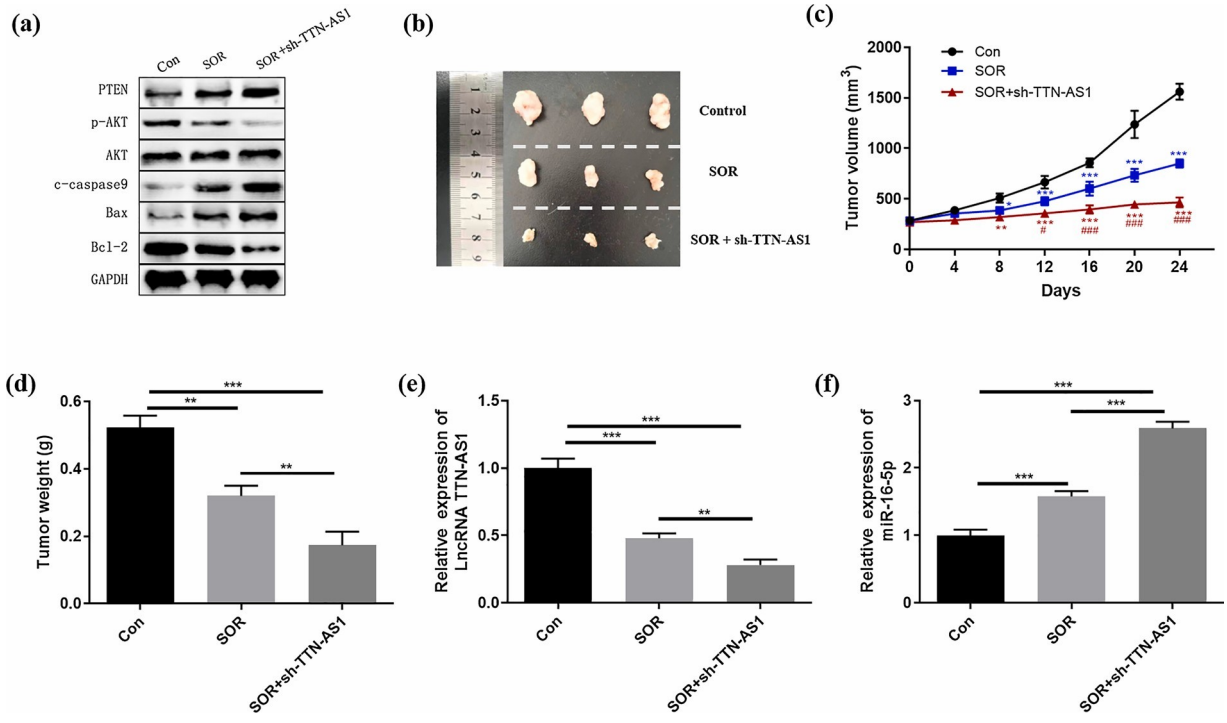

Fig. 6. Knockdown of lncRNA TTN-AS1 alleviated SOR resistance *in vivo*. (a) Western blotting analysis for cleaved caspase 9, Bax, Bcl-2, PTEN, p-Akt, and Akt levels in tumor tissues. (b) Tumor size after sh-TTN-AS1 action. (c) Growth curve of tumor size during 24 days after action, * $P < 0.1$, *** $P < 0.001$ compared with the control group, $^{\#}P < 0.1$, $^{\#\#\#}P < 0.001$ compared with the SOR group. (d) Weight of subcutaneous tumors. (e) qRT-PCR for TTN-AS1 expression in extracted tumor tissues. (f) qRT-PCR for miR-16-5p expression in extracted tumor tissues. ** $P < 0.01$, *** $P < 0.001$.

Author contribution

Yongping Zhou, Yonggang Huang and Xiaoming Ai designed the research; Yongping Zhou, Tu Dai, Zhiyuan Hua, and Jian Xu performed the research; Yongping Zhou, Yuting Lin, Lulu Han, and Xiong Yue analyzed the data; Lichen He and Jinjing Lu contributed the materials; and Yongping Zhou and Xiaoming Ai wrote the paper.

Declaration of Competing Interest

The authors report no declarations of interest.

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